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Probing the Non-Canonical Interface for Agonist Interaction with an α5 Containing Nicotinic Acetylcholine Receptor*

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Abstract

Nicotinic acetylcholine receptors (nAChRs) containing the α5 subunit are of interest because genome-wide association studies and candidate gene studies have identified polymorphisms in the α5 gene that are linked to an increased risk for nicotine dependence, lung cancer, and/or alcohol addiction. To probe the functional impact of an α5 subunit on nAChRs, a method to prepare a homogeneous population of α5-containing receptors must be developed. Here we use a gain of function (9') mutation to isolate populations of α5-containing nAChRs for characterization by electrophysiology. We find that the α5 subunit modulates nAChR rectification when co-assembled with α4 and β2 subunits. We also probe the α5–α4 interface for possible ligand binding interactions. We find that mutations expected to ablate an agonist binding site involving the α5 subunit have no impact on receptor function. The most straightforward interpretation of this observation is that agonists do not bind at the α5–α4 interface, in contrast to what has recently been demonstrated for the α4–α4 interface in related receptors. In addition, our mutational results suggest that the α5 subunit does not replace the α4 or β2 subunits and is relegated to occupying only the auxiliary position of the pentameric receptor.

Keywords

Addiction; Biophysics; Cholinergic receptor; Electrophysiology; Ion channels; Nicotinic Acetylcholine receptors

1. Introduction

Nicotinic acetylcholine receptors (nAChRs) are widespread in the peripheral and central nervous systems. Because these receptors can be activated by nicotine as well as their native ligand acetylcholine, they have been associated with several health-related phenomena.

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Nicotine is the major addictive component of tobacco, and chronic tobacco use (smoking) has been implicated in many types of cancer as well as heart disease. Other related phenomena include an inverse correlation between smoking and Parkinson's disease and the observation that patients with autosomal dominant nocturnal frontal lobe epilepsy who smoke have fewer seizures (Brodtkorb and Picard, 2006).

nAChRs belong to the Cys-loop family of ionotropic receptors, which share a pentameric architecture arranged around a central ion-permeable pore. Many diverse subunit combinations can form functional receptors, and these combinations have distinct pharmacologies concerning responses to acute applications and chronic or repeated applications of nicotinic drugs. Neuronal nAChRs are composed of α2–α11 and β2–β4 subunits and assemble as α plus β or α only pentamers. The neuronal α4β2 receptor subtype is one of the two most abundant nAChRs in the central nervous system (CNS). Two α4β2 pentameric stoichiometries are known: $(\alpha4\beta2)_2(\beta2)$ and $(\alpha4\beta2)_2(\alpha4)$, which shall be referred to as A2B3 and A3B2, respectively (Nelson et al., 2003). Subunit stoichiometry of nAChRs is important in determining pharmacology, stability, and subcellular location. The A2B3 stoichiometry displays higher sensitivity to nicotine and has been proposed to play an especially prominent role in nicotine addiction.

Several brain regions express receptors that contain an α5 subunit (α5* receptors), including the substantia nigra pars compacta, subthalamic nucleus, medial habenula, prefrontal cortex, and hippocampus. Receptors containing α5 play a part in nicotine self-administration and nicotine withdrawal (Fowler et al., 2011). These receptors are also important for dopamine release and attention tasks (Bailey et al., 2010; Gotti et al., 2006; Salminen et al., 2004). The α4β2α5 receptors are more permeable to Ca^{2+} than α4β2 receptors and have a higher sensitivity to nicotine (Kuryatov et al., 2008). The α5 subunit has been assumed to occupy the fifth “auxiliary” position in pentameric receptors, and it has not previously been thought to participate in forming a functional agonist binding site. However, recent studies have proposed that a low affinity binding site exists in the A3B2 α4β2 receptor at the α4–α4 interface (Fig. 1) (Harpsoe et al., 2011; Mazzaferro et al., 2011; Rohde et al., 2012), in addition to the higher affinity binding sites at the α4–β2 interface. This new binding location utilizes an auxiliary subunit interface, leading to questions as to whether α5 can participate in a similar motif.

Given the precise localization and unique functional properties of α5* receptors, α5 presents itself as a valuable therapeutic target. However, currently there are no pharmacological ligands that can functionally isolate α5* receptors. If α5 does participate in a ligand-binding site at the α5–α4 interface, this interaction would be a vital target for selective ligand development.

2. Materials and methods

2.1. Molecular Biology

Mouse nAChR α5 wt, α5-GFP, α4 and β2 subunits were in pGEMhe. The QuikChange protocol (Stratagene) was used for site-directed mutagenesis. Circular DNA for α5, α4 and β2 was linearized as follows: *SphI* restriction enzyme for α5 plasmids and *SbfI* restriction enzyme for the α4 and β2 plasmids. After purification (Qiagen), mRNA was synthesized from linearized DNA template through run-off transcription by using the T7 mMessage Machine kit (Ambion). Purification of mRNA was performed using QIAGEN's RNeasy RNA purification kit.

2.2. Electrophysiology Studies

2.2.1. Xenopus Oocyte Preparation and Injection—*Xenopus laevis* stage V and VI oocytes were harvested via standard protocols (Nowak et al., 1998). The α5 mRNA was mixed with α4 and β2 mRNA in a 10:1:1 ratio by mass and 50 nl were injected into the oocytes delivering 40 ng of total mRNA. After injection, oocytes were incubated at 18° C in ND96+ medium for 24–96 h. The control experiments of only α4 and β2 mRNA with a ratio of 1:1, 1:2 and 10:1 had total mRNA amounts of 6.67 ng, 20 ng and 21 ng, respectively.

2.2.2. Chemical Preparation—Acetylcholine chloride, (−)-nicotine tartrate and mecamylamine hydrochloride were purchased from Sigma-Aldrich and dissolved to 1 M and 0.25 M stock solutions in ND96 Ca^{2+} free buffer (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES at pH 7.5), respectively.

2.2.3. Electrophysiological Experimental Protocols and Recordings—Electrophysiological recordings were performed using two electrode voltage clamp mode with the OpusXpress 6000A (Axon Instruments). The holding potential was −60 mV. All recordings involving α5* receptors and α4β2 receptors used the ND96 Ca^{2+} free solution as the running buffer. All measurements used 1 mL of drug solution applied during 15 s (except 25 s for current-voltage experiments) followed by a 2 min buffer wash except for nicotine application which received a 5 min buffer wash. Dose-response measurements utilized a series of ~3-fold concentration steps, spanning several orders of magnitude, for a total of eight to eighteen doses. Data were low-passed filtered at 5 Hz and digitized at 125 Hz.

Mecamylamine experiments involved three acetylcholine doses, followed by two co-application doses of acetylcholine and mecamylamine, followed by two doses of acetylcholine only. Before each co-application, there was a 30 s pre-incubation of mecamylamine only.

2.3. Data Analysis

2.3.1. Dose-response Analysis—Averaged and normalized data were fit to one or two Hill terms to generate EC_{50} and Hill coefficient (nH) values. All currents for the mecamylamine experiment were normalized to the highest current response pre-mecamylamine addition. The percentage recovery was calculated by comparing pre-and post-mecamylamine applications.

2.3.2. Current-voltage analysis—I–V relations were generated from 400 ms test pulses, applied at intervals of 500 ms, ranging from −110 mV to +70 mV in 20 mV increments. To minimize distortions from desensitization or ion accumulation, the increments proceeded in both depolarizing and hyperpolarizing directions during each drug application, and averaged data were analyzed. To isolate agonist-induced currents, we subtracted records taken in buffer only. The current was averaged during 200 to 400 ms after the jump, and then normalized to the value at −110 mV.

2.3.3. Error Analysis—Error bars on dose-response curves represent standard error of the mean (SEM) values. Maximal current values (wild type vs. V9'S α5 subunit) and voltage jump comparisons (at +70 mV) were subjected to Student t Test analysis and gave t probabilities < 0.001.

3. Results

3.1. Expression of an α5-containing receptor

Accurate interpretation of structure-function relationships from electrophysiological responses requires expression of a homogeneous receptor population. As such, a method to prepare and confirm a homogenous population of α5* receptors must first be established before we can begin to interpret results from mutational analysis of the putative α5–α4 interface. α5 presents a unique challenge because of its role as an accessory subunit. When α5 is co-expressed with α4 and β2 subunits, we consider the possibility of three different receptor populations on the cell surface: $(\alpha4\beta2)_2(\beta2)$, $(\alpha4\beta2)_2(\alpha4)$ and $(\alpha4\beta2)_2(\alpha5)$. The assumption that α4β2α5 receptors have an $(\alpha4\beta2)_2(\alpha5)$ stoichiometry is partly based on analogy to the muscle-type receptor, which contains two conventional binding interfaces (α/γ(ε) and α/δ) and then a single auxiliary subunit, β. Previously, differences in EC_{50} values and rectification behaviors allowed a distinction to be made between the two α4β2 receptor stoichiometries (Xiu et al., 2009). Here, we apply similar strategies to evaluate α5* receptors.

Varying mRNA injection ratios in oocytes can bias assembly to a specific receptor stoichiometry (Moroni et al., 2006; Nelson et al., 2003; Xiu et al., 2009). Specifically, using excess of α5 mRNA compared to α4 and β2 mRNA promotes preferential assembly with an α5 subunit (Ramirez-Latorre et al., 1996). A ratio of 10:1:1 of α5:α4:β2 mRNA was used to bias the system toward incorporation of the α5 subunit. As seen in (Fig. 2), injection of a 1:1 ratio of α4:β2 mRNA produces a biphasic dose-response relation, reflecting the presence of A2B3 (high affinity) and A3B2 (low affinity) forms of the α4β2 receptor. However, a monophasic dose-response relation is clearly seen upon the addition of the α5 mRNA (Ramirez-Latorre et al., 1996). mRNA injections of 1:2 of α4:β2 (resulting in A2B3 receptors) and 10:1 of α4:β2 (resulting in A3B2 receptors) were performed in order to compare EC_{50} values of the two α4β2 receptor stoichiometries with the new value obtained from introducing the α5 mRNA (Fig. 2, Table 1). We find that the EC_{50} value resulting from 10:1:1 α5:α4:β2 mRNA injection is nearly identical to that for the A2B3 receptor. This could indicate that α5-containing receptors coincidentally have nearly the same EC_{50} as A2B3 α4β2 receptors, or that the addition of the α5 mRNA is attenuating the expression of the A3B2 receptor, resulting in a single population of A2B3 receptors at the surface. An additional challenge to evaluating these putative α4β2α5 receptors was the low agonist-induced current (tens to hundreds of nA) seen in these experiments.

To address the first issue, we tested the response to voltage-jump protocols that we have previously used to distinguish A2B3 and A3B2 α4β2 receptors (Xiu et al., 2009). Figure 3 shows that there is a distinct loss of rectification for receptors prepared by a 10:1:1 α5:α4:β2 mRNA injection compared to A2B3 and A3B2 receptors. This result suggests that we have α5* receptors on the surface of the oocyte.

To overcome the small currents that were hampering our efforts to fully characterize these channels, we introduced an often-used reporter mutation at the 9' position of the pore lining M2 helix (Filatov and White, 1995; Kearney et al., 1996; Labarca et al., 1995; Zhong et al., 1998). Mutations in this region frequently result in increased expression levels. In addition, 9' mutations typically cause a gain of function in the receptor, evidenced by a reduction in EC_{50} values and due to an altered P_{open}, without affecting the ligand-binding domain (Gleitsman et al., 2009). Most subunits of nAChRs have a conserved Leu at the 9' position, but the accessory subunits α5 and β3 contain a Val in that position. The V9'T (Groot-Kormelink et al., 2001) or V9'S (Li et al., 2011) mutation has been employed to study α3β4α5 nAChRs, but not α4β2α5 nAChRs. In the present work a V9'S mutation was introduced into the α5 subunit, and the above experiments were repeated with the α5V9'S

subunit. Interestingly, we observe nearly identical EC_{50} values for $(\alpha4\beta2)_2(\alpha5)$ and $(\alpha4\beta2)_2(\alpha5V9'S)$ as shown in (Fig. 2). However, a substantial increase in agonist-induced current was observed (Table 1), and more consistent responses among batches of oocytes were noted. These observations further support the notion that the α5 subunit has been incorporated, since the only change between the experiments was the mutation in the α5 subunit.

3.2. The α5V9'S mutation confers distinct physical properties allowing for definitive establishment of the subunit's incorporation

The two stoichiometries of the α4β2 receptor are distinguishable by their distinct EC_{50} values. However, their rectification properties are similar (Fig. 2,3, Table 1,2). Previous studies have shown that introduction of an L9'A mutation in the α4 subunit changes the rectification behavior of α4β2 receptors (Xiu et al., 2009). Xiu *et al*. report that the A2B3 receptor rectifies much more markedly than the A3B2 when the a4 L9'A mutation is present (Xiu et al., 2009). Similarly, we find that introduction of the V9'S mutation to the α5 subunit produces a more marked loss of rectification compared to the wild type α5 subunit (Fig. 3). In anticipation of future studies on receptor trafficking and localization, we prepared receptors with the V9'S mutation and meGFP inserted in the M3–M4 loop of the α5 subunit. This receptor also gives a wild type EC_{50} and a loss of rectification (Table 1,2).

In addition to the EC_{50} and rectification data, we also sought to more fully characterize the $(\alpha4\beta2)_2(\alpha5V9'S)$ receptor. Mecamylamine has been extensively characterized as an open channel blocker with a slow wash off time for the α4β2 receptor (Papke et al., 2001). A distinctive property of this open channel blocker is its “trapping” behavior: it associates and disassociates preferentially from the open pore of the receptor (Lester, 1992). Because of the prolonged wash off time, subsequent applications of an agonist are generally needed to dissociate the molecule from the pore.

The results in Figure 4 show a dramatic difference between the $(\alpha4\beta2)_2(\alpha5V9'S)$ receptor and the α4β2 receptor. When the α5V9'S subunit is present, mecamylamine washes off the receptor completely within 120 s. We also find that introducing an L9'A mutation into the α4 subunit results in mecamylamine successfully washing out of both the A2B3 and A3B2 receptors (Table 3). This suggests that the observed results with the α5V9'S subunit are due to the 9' mutation rather than an intrinsic property of the subunit. However, this still generates an important observation for this system. For studies of the $(\alpha4\beta2)_2(\alpha5V9'S)$ receptor, the observation of nearly complete response recovery within 120 s indicates that we have prepared a receptor population on the plasma membrane that is highly enriched in α5* receptors, if not completely homogeneous. If a more mixed population containing both α5* and α4β2 receptors was being expressed, the response would have recovered less fully. Results from the rectification and open channel blocker experiments strongly indicate that we are able to express on the oocyte surface an α5* receptor population that is homogeneous or very nearly so. With this, we can now begin to investigate the α5–α4 interface, knowing that responses from introduced mutations are directly due to changes in the $(\alpha4\beta2)_2(\alpha5V9'S)$ receptor.

3.3. Mutational analysis of the aromatic box at the α5–α4 interface showed no functional impact

The aromatic box of nAChR agonist binding sites is highly conserved and has been extensively characterized (Cashin et al., 2005; Dougherty, 2008; Mu et al., 2003; Puskar et al., 2011; Tavares et al., 2012)}. The aromatic box mediates an essential cation-π interaction between the positively charged portion of the ligand and one (or occasionally two) of the five aromatic residues (A, B, C1, C2, and D) of the aromatic box. In fact, this aromatic box

structure has been seen in a wide range of binding sites for cationic structures (Dougherty, 2013). The sequence alignment in Figure 1 highlights the residues of interest for studying the α5 subunit. Residues TrpB, TyrC2, and TyrA have previously been shown to be involved in cation-π interactions in other receptors, and these residues would be part of the principal component of an aromatic box contributed by α5 if an α5–α4 binding site exists. The TrpD residue of the α5 subunit was also investigated to probe the possibility of agonist binding at the β2–α5 interface. In addition, this mutation can subsequently probe the possibility that α5 subunits were replacing β2 subunits that would normally participate in the α4–β2 binding site. Interestingly, the C1 residue of the aromatic box that is typically a Tyr in α subunits is an Asp in the α5 subunit.

An alanine scan of the aromatic residues of α5 was performed. As shown in Table 4, negligible changes in EC_{50} were seen in all cases for both ACh and nicotine as agonists. Since the C1 site is not aromatic in the α5 subunit, an Asp-to-Tyr mutation was introduced. Again, no change in EC_{50} was seen. Current-voltage relations confirmed the inclusion of the α5 subunit for all the mutations (**data not shown**).

4. Discussion

Accessory subunits play important roles in nAChR function, because they confer unique properties to their parent receptors. Not surprisingly, expression of these subunits is highly regulated and restricted to specific brain regions (Gotti et al., 2006; Miwa et al., 2011). Some examples of this region specificity are found in the cerebral cortex, where α5* receptors are expressed only in layer VI, and in the striatum, where α5 may be expressed only in the dopaminergic neurons of the caudatoputamen, but not in the nucleus accumbens region (Exley et al., 2012; Salas et al., 2003; Wada et al., 1990). α5 is of particular interest because well-replicated human genome-wide association studies have identified a single nucleotide polymorphism that affects the risk for nicotine dependence, lung cancer, and alcohol dependence (Bierut, 2010; Hartz et al., 2012; Saccone et al., 2009). This mutation, encoding Asn at position 398 in the coding region of the *CHRNA*5 gene, also affects nicotine self-administration in mice (Fowler et al., 2011; Frahm et al., 2011). Thus, it would be beneficial to be able to probe functional differences of α5* receptors using pharmacological agents *in vivo*.

Here we aimed to elucidate possible ligand binding motifs involving the α5 subunit in the $(\alpha4\beta2)_2(\alpha5)$ receptor. The α designation for a5 arose from the existence of adjacent Cys residues in the C loop (Fig. 1B), although other aspects of the C loop such as replacement of conserved TyrC1 and residue deletions are more β-like. Also, α5 is unable to form functional receptors unless other α subunits are also expressed. As such, α5 is generally considered to be an accessory subunit. However, the discovery of an α4–α4 binding interface in A3B2 α4β2 receptors suggests the possibility of an unusual binding site at the α5–α4 binding interface (Mazzaferro et al., 2011).

To address this question, we first optimized our expression system to ensure a homogeneous, or at least very highly enriched, population of receptors. Early studies on wild type receptors in oocytes suggested that biasing mRNA injection ratios strongly toward α5 would produce a homogeneous population of α5-containing receptors. However, expression levels were low, making thorough characterization challenging. Introducing a V9'S mutation into the α5 subunit resulted in increased agonist responses, allowing greater consistency and reproducibility between experiments.

We have two lines of evidence to support the argument that α5 is incorporated into the receptor and that the population of receptors on the oocyte plasma membrane is

homogeneous or very nearly so. First, we see altered rectification behavior for channels with α5 vs. pure α4β2 receptors (Fig. 3). The effect is evident in fully wild type receptors, but is more apparent for the α5 V9'S receptors. This is the second system for which we have observed that a 9' mutation can markedly affect channel rectification properties. A single 9' mutation in the accessory subunit location – α5V9'S in this case – is sufficient to alter the rectification properties of the receptor. In the α4β2 receptor, the A3B2 receptor with the L9'A mutation in the α4 subunit shows a marked loss of rectification, but replacing the third α4 L9'A subunit with a β2 subunit (A2B3) restores rectification (Table 2) (Xiu et al., 2009).

The second argument for incorporation of α5 into our expressed receptors is based on altered behavior by the channel blocker mecamylamine. For α4β2 receptors, mecamylamine blockade washes out very slowly, and most efficiently when agonist is also added (Fig. 4B). When the α5 V9'S subunit is included, mecamylamine blockade washes out readily. Note that the washout is essentially complete, arguing that all, or very nearly all, receptors contain the α5 subunit. Any population of α4β2 receptors would have led to residual mecamylamine block. The alteration of mecamylamine block is only seen when the V9'S mutation is present in α5. An α4 L9'A mutation also impacts mecamylamine block, indicating that it is the pore mutation that is affecting block, not the intrinsic properties of the α5 subunit. Presumably, mecamylamine binds within the pore near the 9' residue.

Having established that α5 is incorporated into the receptors, we can comment on its impact on receptor function. It is interesting that the α4β2α5 receptor has essentially the same EC_{50} as the $(\alpha4\beta2)_2(\alpha4)$ receptor. This strongly suggests that the α5 subunit is not displacing an α4 subunit that contributes to an agonist binding site, as this should produce an EC_{50} change, especially since any mutation of TyrC1 in nAChR agonist binding sites shifts EC_{50} strongly, and α5 is mutated at that site. The same argument could be made that α5 does not replace a β2 subunit that contributes to the agonist binding site, although perhaps less forcefully since the two have similar sequences in loop D. Note that if two (or more) α5 subunits were incorporated, then one of the canonical α4/β2 interfaces would disappear, and again it is difficult to imagine that happening without EC_{50} being impacted. It is surprising that the V9'S mutation in α5 does not shift EC_{50}. Typically, introducing a polar substituent at any 9' position of an nAChR leads to a drop in EC_{50}. In other cases the wild type 9' residue is Leu not Val, but it is not obvious why that would lead to a change in behavior

To probe for the existence of an agonist binding site at the α5–α4 interface, we mutated the conserved residues in α5 that would contribute to the aromatic box of such a binding site (TyrA, TrpB, TyrC1, and TyrC2). Converting an aromatic to an Ala or converting the Asp that aligns with TyrC1 to Tyr did not have a marked affect on agonist responses. These are fairly dramatic mutations that would produce very substantial shifts in EC_{50} in established agonist binding sites. We also mutated TrpD of α5 to Ala, to probe whether α5 replaces a β2 subunit and contributes a complementary face, interacting with an α4 subunit. Again, no meaningful impact on receptor function was seen.

The most straightforward interpretation of these results is that there is no ACh or nicotine binding site at the α5–α4 interface, and so mutation of key residues has no functional impact. We cannot rule out the possibility that ACh binds to the α5–α4 interface, but that the binding does not meaningfully impact receptor function. Also, our assay precludes the application of drugs at concentrations greater than 100 μM for α5* receptors, and so it is possible that there is a very low affinity binding site (dissociation constant on the order of mM). However, the biological implications of such a site seem negligible. Also, it is possible that other drugs could bind at the α5–α4 interface, and that binding at the α5–α4 interface could have a functional consequence in receptors in which the conventional, α4–β2 interfaces have in some way been compromised.

Although it does not contribute an ACh binding site in the nAChRs studied here, the α5 subunit can play other important roles. The α5 subunit also co-assembles with α3 and β4 subunits to form functional nAChRs, especially in peripheral ganglia and in the medial habenula-interpeduncular nucleus pathway (Fowler et al., 2011), and it has been shown to modulate expression levels of these receptors (George et al., 2012). Also, the α5 subunit could impart pore-related differences such as Ca^{2+} permeability (Kuryatov et al., 2008), and intracellular loop-related differences such as endoplasmic reticulum exit and synaptic targeting (Gotti et al., 2005).

In conclusion, we have developed a protocol for preparing highly enriched populations of $(\alpha4\beta2)_2(\alpha5)$ nAChRs, and we have shown that mutations that would be expected to disrupt an α5–α4 interfacial binding site do not affect receptor function. In addition, we have shown that α5 subunits only occupy the auxiliary position when co-assembled with α4 and β2 subunits. Further studies will be required to develop a strategy for selectively probing α5* receptors with pharmacological agents.

Acknowledgments

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Abbreviations

nAChR	nicotinic acetylcholine receptor
CNS	central nervous system
nH	Hill coefficient
A2B3	high sensitivity $(\alpha4\beta2)_2(\beta2)$ receptor
A3B2	low sensitivity $(\alpha4\beta2)_2(\alpha4)$ receptor
SEM	standard error of the mean

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Highlights

- α4β2α5 receptors modulate rectification properties compared to α4β2 receptors
- α5V9'S allows generation and observation of a homogenous receptor population
- Mutational analysis shows no functional ligand binding site at the α5–α4 interface

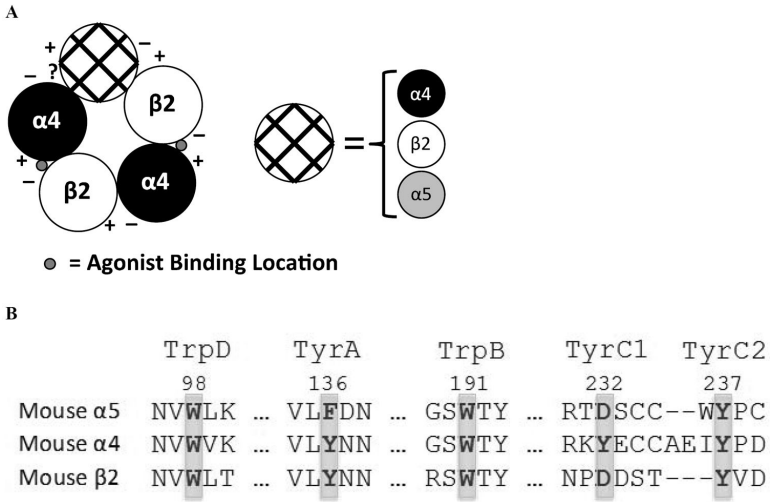

Fig. 1.

A. View of nAChRs from the extracellular solution. The α4β2 receptor has two α4 subunits, two β2 subunits, and two conventional antagonist/agonist binding sites, at α4–β2 interfaces. The fifth subunit is the "accessory" subunit, and in this study three possibilities are the α4, α5 or β2 subunits. The accessory subunit may also contribute to a binding interface, shown by "?" B. Sequence alignment of the mouse nAChR subunits highlighting the aromatic box residue locations on loops D, A, B, and C (in sequence). The residues shown are identical to their human sequence except the final residue (Cys) in the mouse α5 subunit is a tyrosine (Tyr, Y) in the human subunit. Note that TrpD is on the "complementary" face of a subunit, and so it is not expected to contribute to an α5–α4 interface.

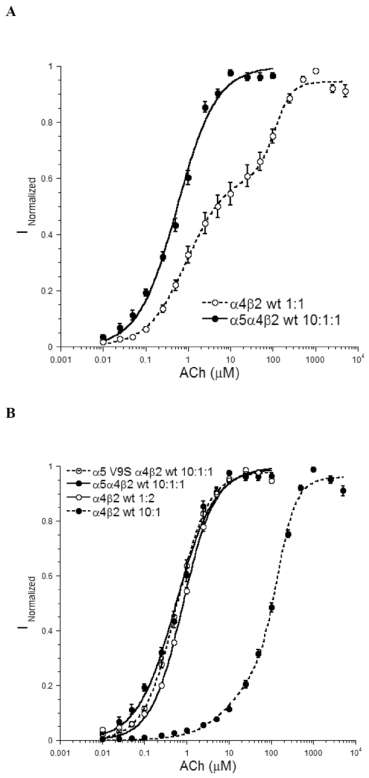

Fig. 2.

A. Dose response relations for the mouse α4β2 receptor with a 1:1 injection ratio, and for the mouse $(\alpha4\beta2)_2(\alpha5)$ receptor with a 10:1:1 α5:α4:β2 mRNA injection ratio. B. Comparison of dose-response relations for the two stoichiometries of the mouse α4β2 receptors (A2B3 and A3B2) and the mouse $(\alpha4\beta2)_2(\alpha5)$ receptor with and without the α5-V9'S mutation.

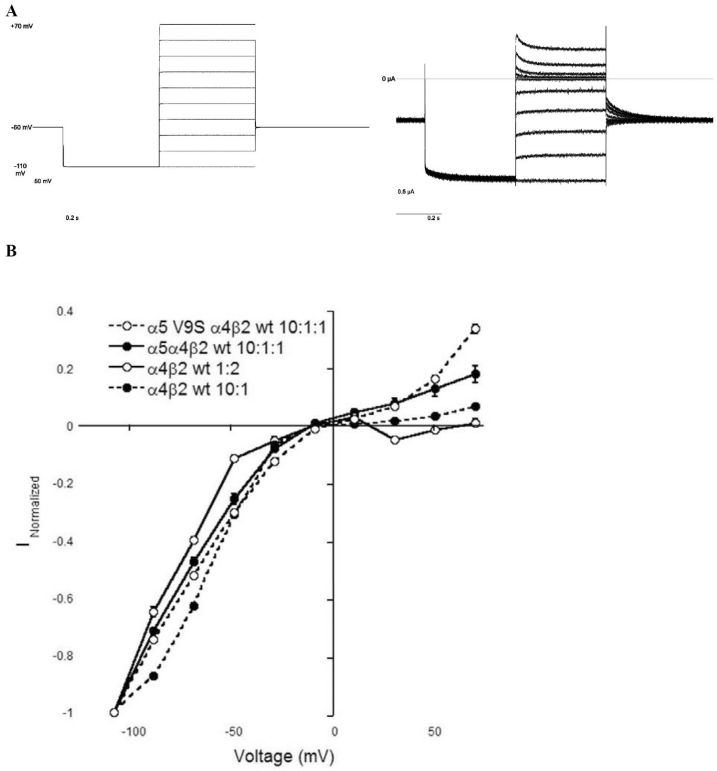

Fig. 3.

Summary of rectification analysis. A. Sample traces of the voltage jump experiment. The superimposed command voltage sweeps (left) and the ACh-induced currents (right) for the $(\alpha4\beta2)_2(\alpha5V9'S)$ receptor are shown. B. Comparison of current-voltage relations for the two stoichiometries of the mouse α4β2 receptors (A2B3 and A3B2) and the mouse $(\alpha4\beta2)_2(\alpha5)$ receptor with and without the 9' mutation.

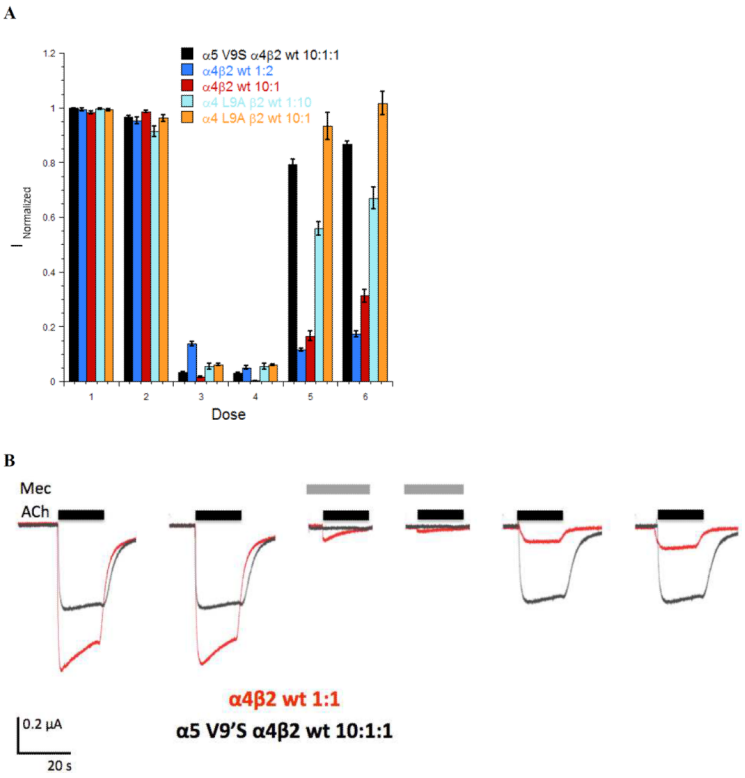

Fig. 4.

A. Response recovery following co-application of acetylcholine and mecamylamine following the protocol in part B. B. A sample current trace of a single oocyte recording for each of the subunit combinations indicated. The α4β2 nAChR is in red and the $(\alpha4\beta2)_2(\alpha5V9'S)$ nAChR is in black. A 120 s wash period occurred between each dose.

Table 1

EC_{50} values, Hill coefficients, and current ranges for receptors studied here.

Receptor (Ratio)	EC_{50} (μM)	Hill	n	Current Range (μA)
α4 wt β2 wt (1:1)	0.9 ± 0.2 (low)	0.9 ± 0.1 (low)	25	−0.14 to −7.0
	110 ± 15 (high)	2.1 ± 0.5 (high)	-	-
α4 wt β2 wt (1:2)	0.80 ± 0.05	1.15 ± 0.7	13	−0.15 to −0.97
α4 wt β2 wt (10:1)	141 ± 27	2.0 ± 0.7	17	−1.5 to −16
α4L9'A β2 wt (1:10)	0.39 ± 0.02	1.25 ± 0.7	13	−0.31 to −7.2
α4L9'A β2 wt (10:1)	0.046 ± 0.001	1.27 ± 0.04	13	−4.1 to −22
α5 wt α4 wt β2 wt (10:1:1)	0.55 ± 0.05	0.95 ± 0.1	12	−0.027 to −0.20
α5V9'S α4 wt β2 wt (10:1:1)	0.57 ± 0.01	1.14 ± 0.01	26	−0.38 to −5.7
α5V9'S GFP α4 wt β2 wt (10:1:1)	0.76 ± 0.02	1.07 ± 0.03	17	−0.34 to −7.2

Agonist = Acetylcholine

Table 2

Voltage jump results for receptors studied here.

Receptor (Ratio)	ACh Induced Current at +70 mV (Normalized)	Current Range (μA) at −110mV	Current Range (μA) at +70mV	n
α4 wt β2 wt (1:1*)	0.01 ± 0.01	−0.35 to −1.8	−0.02 to 0.11	34
α4 wt β2 wt (1:2)	0.01 ± 0.01	−0.10 to −1.4	−0.03 to 0.09	34
α4 wt β2 wt (10:1)	0.067 ± 0.008	−1.7 to −24	0.08 to 2.0	60
α4L9'A β2 wt (1:10)	0.054 ± 0.005	−0.23 to −5.0	−0.006 to 0.25	28
α4L9'A β2 wt (10:1)	0.34 ± 0.01	−1.0 to −18	0.39 to 6.4	31
α5 wt α4 wt β2 wt (10:1:1)	0.10 ± 0.03	−0.073 to −0.14	−0.013 to 0.027	6
α5V9'S α4 wt α2 wt (10:1:1)	0.34 ± 0.02	−0.56 to −2.5	0.15 to 1.2	34
α5V9'S GFP α4 wt β2 wt (10:1:1)	0.33 ± 0.02	−0.71 to −1.9	0.27 to 0.5	12

ACh doses were the corresponding receptor's EC50 value for the voltage jump experiments

* The smaller of the two EC50 value was used for this measurement

Table 3

Results of mecamylamine experiments.

Receptor (Ratio)	Signal Recovery	Current Range (μA)	n
α5V9'S α4 wt β2 wt (10:1:1)	80 ± 2 %	−0.46 to −1.0	14
α4 wt β2 wt (1:2)	12 ± 1 %	−0.16 to −1.5	13
α4 wt β2 wt (10:1)	17 ± 2 %	−1.3 to −22	16
α4L9'A β2 wt (1:10)	56 ± 2 %	−0.08 to −0.84	15
α4L9'A β2 wt (10:1)	93 ± 5 %	−1.3 to −20	14

ACh doses were 10 times the corresponding receptor's EC50 value for the mecamylamine experiments Mecamylamine was kept at constant 100 μM value to ensure full channel block

Table 4

Mutagenesis results for various α5-containing receptors.

Receptor (Ratio)	*Acetylcholine* EC50 (μM)	Hill	n	Fold Shift	Current Range (μA)	*Nicotine* EC50 (μM)	Hill	n	Fold Shift	Current Range (μA)
α4 wt β2 wt (1:2)	0.80 ± 0.05	1.2 ± 0.1	13	-	0.15 – 0.97	0.17 ± 0.01	1.4 ± 0.1	15	-	0.07 – 0.45
α4 wt β2 wt (10:1)	140 ± 30	2.0 ± 0.7	17	-	1.5 – 16	6.7 ± 1	0.86 ± 0.1	12	-	0.39 – 3.3
α5V9'S α4 wt β2 wt (10:1:1)	0.57 ± 0.01	1.1 ± 0.01	26	-	0.38 – 5.7	0.19 ± 0.02	1.4 ± 0.2	34	-	0.11 – 1.4
α5V9'S W98A (Trp D)	1.1 ± 0.08	1.1 ± 0.07	10	**1.9**	0.038 – 1.5	0.27 ± 0.02	1.4 ± 0.1	15	**1.4**	0.030 – 0.65
α5V9'S F136A (Tyr A)	0.66 ± 0.02	1.2 ± 0.04	10	**1.2**	0.21 – 0.54	0.16 ± 0.02	1.3 ± 0.2	12	**0.8**	0.095 – 0.30
α5V9'S W191A (Trp B)	0.55 ± 0.03	1.2 ± 0.08	19	**1**	0.068 – 0.67	0.16 ± 0.01	1.4 ± 0.1	13	**0.8**	0.092 – 1.8
α5V9'S D232Y (Tyr C1)	0.60 ± 0.02	1.3 ± 0.04	13	**1**	0.20 – 1.2	0.19 ± 0.02	1.4 ± 0.2	12	**1**	0.56 – 3.3
α5V9'S Y237A (Tyr C2)	0.82 ± 0.03	1.2 ± 0.05	16	**1.4**	0.067 – 0.41	0.17 ± 0.01	1.3 ± 0.1	10	**0.9**	0.41 – 3.1